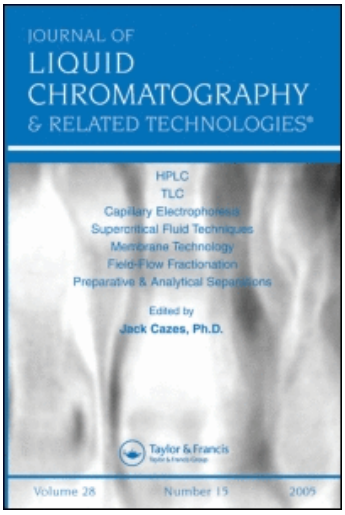

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Comparative Study of the Retention Behavior of Lipidic Peptides on RP-18 and Supelcosil™ LC-ABZ Stationary Phases

D. Korakas[ab]; K. Valkó[ac]

[a] Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, London, United Kingdom [b] Department of Chemistry, Section of Organic and Biochemistry, University of Ioannina, Ioannina, Greece [c] Department of Physical Sciences, Langley Court, United Kingdom

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COMPARATIVE STUDY OF THE RETENTION BEHAVIOR OF LIPIDIC PEPTIDES ON RP-18 AND SUPELCOSIL™ LC-ABZ STATIONARY PHASES

D. KORAKAS[1] AND K. VALKÓ[2]*
Department of Pharmaceutical Chemistry
School of Pharmacy
University of London
29-39 Brunswick Square
London WC1N 1AC, United Kingdom

ABSTRACT

A series of lipidic amino acids and peptides were investigated by systematic change of the mobile phase composition using traditional octadecylsilica stationary phase and the newly developed Supelcosil™ LC-ABZ column. The mobile phases contained various concentrations of methanol and acetonitrile combined with 0.1% trifluoroacetic acid (TFA). The log k′ values were plotted as a function of organic phase concentration in the mobile phase. In some cases parabolic curves were obtained on the traditional octadecyl silica stationary phase due to the presence of the free silanol groups, while on the Supelcosil™ LC-ABZ column straight lines were obtained. Much lower concentration of organic phase was needed for getting similar retention times on the Supelcosil™ LC-

[1]On leave from University of Ioannina, Department of Chemistry, Section of Organic and Biochemistry, 451 10 Ioannina, Greece
[2]Present address: Department of Physical Sciences, Langley Court, Beckenham, BR3 3BS United Kingdom

ABZ column, so no retention was observed at higher organic phase concentrations where the silanol effect could have been observed. The slope and the intercept values of the linear section of the log k' vs organic phase concentration lines were calculated and the chromatographic hydrophobicity index (φ_0) was also expressed for each compound. By the help of the log k' vs organic phase concentration plots the optimum conditions for the separation of diastereomeric isomers were predicted.

INTRODUCTION

Lipidic amino acids and peptides were suggested as possible conjugates to pharmacologically active compounds by Gibbons et. al. [1] and Sakarellos et. al. [2]. They are expected to possess a degree of membrane-like character which is sufficient to facilitate the passage of poorly absorbed drugs across biological membranes to reach their active sites. Quantitative structure activity relationships were revealed for a series of lipidic amino acid conjugates of ß-lactame antibiotics [3] which showed the importance of the lipidic side chain in the in vivo action. The antigenic role of single residues and the role of the lipidic side chain to facilitate the formation of the ß-folding have been also investigated for the lipidic peptides studied [4]. The compounds investigated are might serve as possible conjugates to pharmacologically active molecules. The 10 peptide (compound 5) was found to be active on Torpedo acetylcholine receptor. Its modification (compound 6 and 7) might results in more active compounds.

As the synthesis of lipidic amino acid derivatives always results in diastereomeric mixtures, the separation of the isomers is of great importance. Very probably the biological activity of the isomers is different. This is to be measured after preparative HPLC separation of the isomers.

As the lipidic amino acids and peptides have amphophilic character (cationic and anionic plus long hydrophobic side chain) they can be regarded as "difficult" compounds in the chromatography. High performance liquid chromatography can be very useful not only for the final purification of compounds and analytical quality control but also for the separation of diastereomeric isomers which always occur during the synthesis. The Supelcosil™ LC-ABZ column was designed to be suitable for analyses of acidic, basic, zwitterionic, and neutral compounds, without the use of silanol-suppressing mobile phase conditions and additives. As the compounds are soluble in aqueous solutions and they are zwitterions the newly developed Supelcosil™ LC-ABZ column seemed to be a promising stationary phase. For revealing the similarities and dissimilarities between the traditional octadecylsilica phases and the new one the retention of the compounds expressed by log k′ values were measured as a function of methanol, acetonitrile, acetonitrile TFA concentration.

EXPERIMENTAL

The compounds were synthesized as it was described by Gibbons et. al. [1] and Sakarellos et. al. [2]. They were chromatographically pure. The chemical structure of the compounds can be seen in Table 1. Under some HPLC conditions, the D, L diastereomers of compound 7 were separated and denoted as 7a and 7b for the first and the later eluting peaks, respectively.

The HPLC measurements were carried out on a Gilson HPLC system consisting of Model 303 pumps, Model 115 variable wavelength UV detector and Rheodyne injector (Anachem, Luton, U. K.). Samples were dissolved 1 mg/ml concentration in water or aqueous methanol and 20 μl was

TABLE 1.

No.	Structure of the Compound
1.	Boc-NH-CH(CH_2)$_{13}CH_3$-CONH(CH_2)$_{13}CH_3$
2.	HCl-H_2N-CH(CH_2)$_{13}CH_3$-CONH(CH_2)$_{13}CH_3$
3.	HCl-H_2N-CH(CH_2)$_3CH_3$-CONHCH(CH_2)$_{13}CH_3$-$COOCH_3$
4.	Boc-NH-CH(CH_2)$_3CH_3$-CONHCH(CH_2)$_{13}CH_3$-$COOCH_3$
5.	Trp-dAsn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys
6.	Trp-Asn-Pro-Ala-dAsp-Tyr-Gly-Gly-Ile-Lys
7	Trp-Asn-Pro-Gly-Asp-Tyr-Gly-Gly-Ile CO-NH-(D,L)CH(CH_2)$_{10}CH_3$-COOH

injected onto the column. Detection was carried out at 210 nm (0.1 AUFS). Spherisorb ODS 5 μm (4.6 x 50 mm) column (PhaseSep, Deeside, U. K.) and Supelcosil™ LC-ABZ (4.6 x 150 mm) (Supelco Inc., Bellefonte, PA, U. S. A.) columns were used. The mobile phases were various concentration of methanol - water (mobile phase A: MeOH ranging 95 - 35 %), acetonitrile - water (Mobile phase B: AcN ranging 95 - 15%), acetonitrile, 0.1% TFA - water mixtures (mobile phase C: AcN ranging 95 - 15%). The flow rate was always 1 ml/min.

The retention time measurements were repeated three times consecutively and the average was taken into account in the calculations of the capacity ratio (k'). The first solvent peak was regarded to be the dead time.

RESULTS AND DISCUSSION

The log k' values of all of the 8 compounds were calculated at various concentration of methanol,

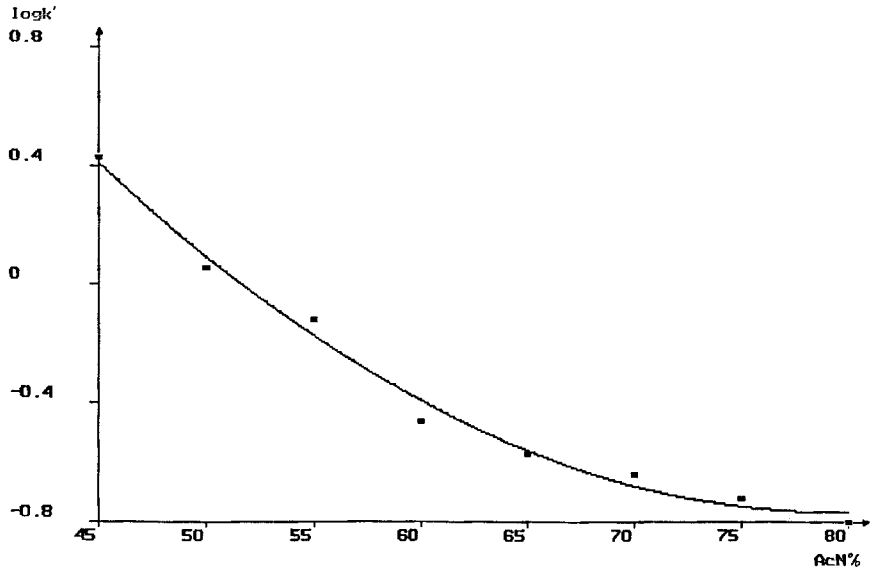

FIGURE 1.The plot of the log k' vs acetonitrile concentration obtained on Spherisorb ODS column for compound 7. The mobile phase also contained 0.1% TFA.

acetonitrile, acetonitrile and 0.1% TFA mixtures. The obtained log k' values were plotted as a function of the organic phase concentration and the slope and the intercept values of the straight lines were calculated. For some derivatives (compound 7) parabolic curves were obtained on Spherisorb ODS stationary phase as it can be seen in Figure 1. This finding is due to the residual silanol groups on the stationary phase [5]. In those cases only the linear section of the curve at lower organic phase concentration was considered in calculation of the slope (S) and intercept (log k'_0) straight values to achieve correlation coefficients always higher than 0.99. The slope (S) and the intercept (log k'_0) values are listed in Table 2 and 3 on the Spherisorb and

TABLE 2.

The slope (S) and the intercept ($\log k'_0$) values of the straight line obtained on Sperisorb ODS column by changing the methanol, acetonitrile (with and without 0.1% TFA) concentration.

No.	$\log k'_{0MEOH}$	S_{MeOH}	$\log k'_{0ACN}$	S_{ACN}	$\log k'_{0ACNTFA}$	S_{ACNRFA}
1.	3.0056	-0.0402	1.2029	-0.0181	0.9898	-0.0190
2.	2.2519	-0.0368	0.9183	-0.0138	1.3440	-0.0261
3.	2.1203	-0.0336	1.0999	-0.0167	1.1013	-0.0215
4.	1.2716	-0.0188	1.0489	-0.0160	1.0867	-0.0211
5.	2.6764	-0.0500	0.7850	-0.0115	0.3014	-0.0118
6.	2.9925	-0.0569	0.8457	-0.0129	0.4254	-0.0138
7a	2.4550	-0.0447	1.9078	-0.0279	2.6389	-0.0504
7b	2.3720	-0.0431	1.9078	-0.0279	2.4741	-0.0435

TABLE 3.

The slope (S) and the intercept ($\log k'_0$) values of the straight line obtained on Supelcosil™ LC-ABZ column by changing the methanol, acetonitrile (with and without 0.1% TFA) concentration.

No.	$\log k'_{0MEOH}$	S_{MeOH}	$\log k'_{0ACN}$	S_{ACN}	$\log k'_{0ACNTFA}$	S_{ACNRFA}
1.	0.8635	-0.0170	0.7706	-0.0144	1.3116	-0.0271
2.	0.8890	-0.0182	1.0591	-0.0184	1.2332	-0.0258
3.	0.9639	-0.0203	0.6847	-0.0116	1.4809	-0.0293
4.	0.8691	-0.0175	0.7638	-0.0133	1.0173	-0.0238
5.	3.0629	-0.0636	1.2290	-0.0180	2.0698	-0.1052
6.	2.6267	-0.0522	1.4029	-0.0195	2.0190	-0.0982
7a	3.3949	-0.0491	2.1530	-0.0237	4.1595	-0.1045
7b	3.5253	-0.0496	2.1012	-0.2148	4.3605	-0.1025

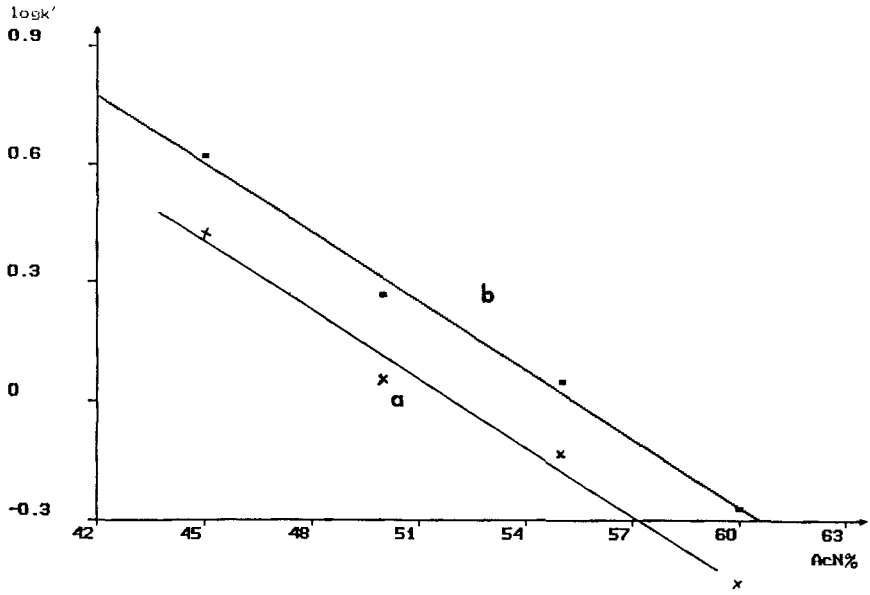

FIGURE 2. The "linear" portion of the plot of the log k' vs acetonitrile concentration obtained on Spherisorb ODS column for compounds 7. The mobile phase contained 0.1% TFA.

Supelcosil column, respectively. Figure 2 shows the linear section of log k' vs acetonitrile concentration plots for compound 7a and 7b on Spherisorb ODS column. It can be seen that the largest difference in the log k' values could be obtained at higher acetonitrile concentration (around the minimum area of the parabolic curve). Figure 3 shows the same plot obtained on Supelcosil™ LC-ABZ column. The opposite trend could be observed. The separation of the diastereomeric isomers takes place at lower acetonitrile concentration, the difference between the log k' values are the largest. It is also noticeable, that much lower acetonitrile concentration can be used with Supelcosil™ LC ABZ column

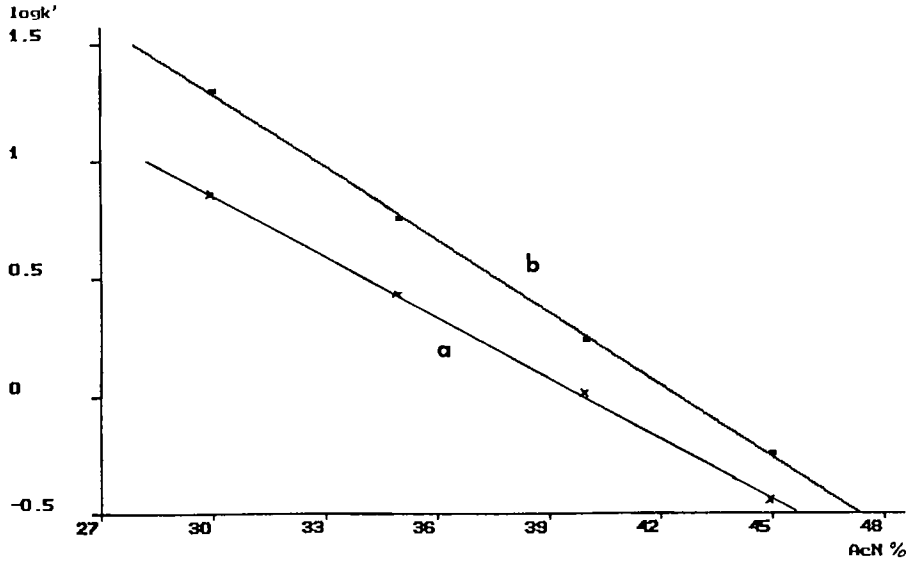

FIGURE 3.The plot of the log k′ vs acetonitrile concentration obtained on Supelcosil^{TM} LC-ABZ column for compound 7.

to obtain the same log k′ region. This means that the stationary phase itself is less hydrophobic and retain less the compounds. It is also very interesting that the slope of the log k′ vs AcN concentration plots are much higher on Supelcosil^{TM} LC-ABZ column than on the Spherisorb ODS for compounds 5 - 7 containing longer peptide residues.

Compound 5 and 6 differs from each other only with the optical isomers of two amino acids. They could be separated only on the Supelcosil^{TM} LC-ABZ column. The separation factor values (α) as a function of the acetonitrile concentration curves on Spherisorb ODS and Suplecosil^{TM} LC-ABZ column are shown in Figure 4 and 5, respectively. Again slightly better (never base line)

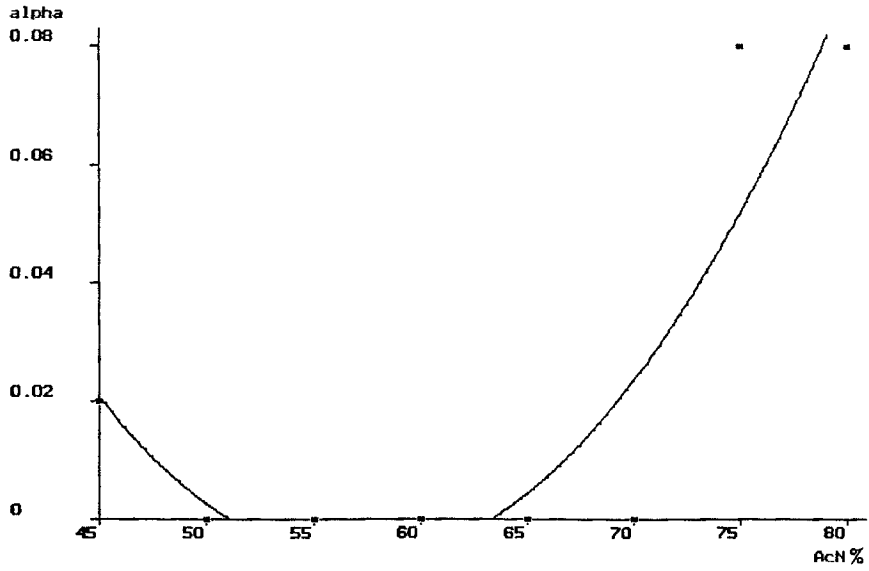

FIGURE 4. The plot of the separation factor (α) vs acetonitrile concentration obtained on Spherisorb ODS column for the racemic mixture of compounds 5 and 6. The mobile phase contained 0.1% TFA.

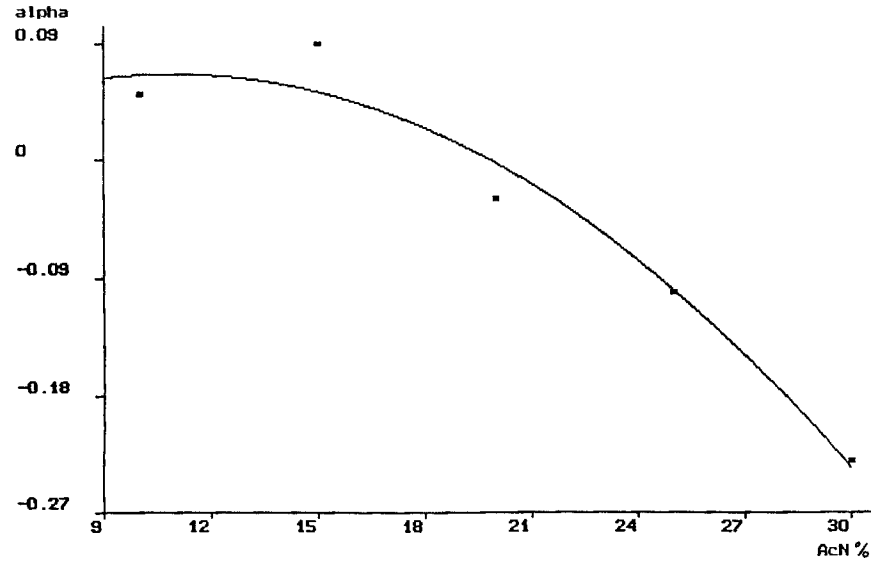

FIGURE 5. The plot of the separation factor (α) vs acetonitrile concentration obtained on Supelcosil™ LC-ABZ column obtained for the racemic mixture of compound 5 and 6. The mobile phase contained 0.1% TFA.

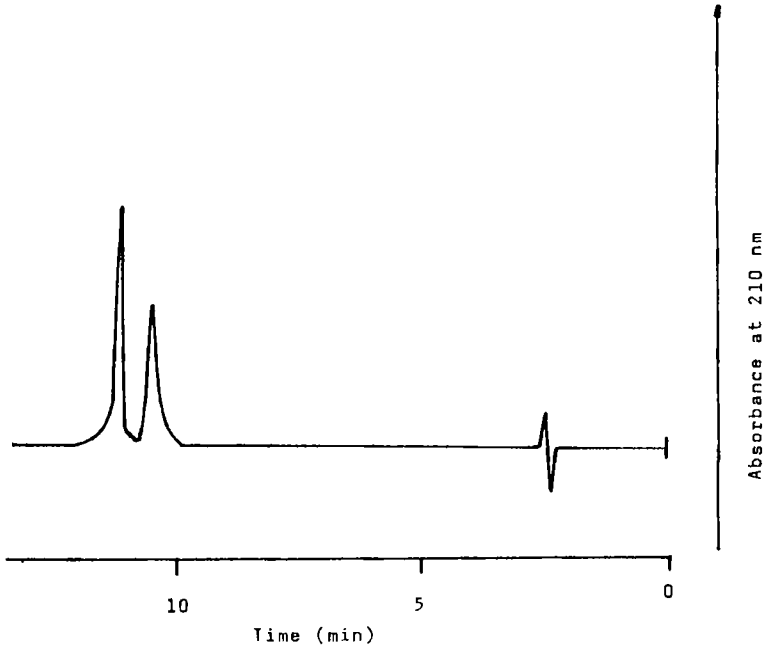

FIGURE 6. The chromatogram obtained by injecting the diastereomeric mixture of compound 5 and 6 on Supelcosil™ LC-ABZ column. The mobile phase 15% acetonitrile, 0.1% TFA; the flow rate was 1.00 ml/min; detection was carried out at 210 nm. 20 μl was injected from the solution containing 0.5 mg/ml of each compound.

separation could be observed at higher acetonitrile concentration on the Spherisorb column. Beautiful base line separation of the two isomers on the Supelcosil™ LC-ABZ column could be achieved as it is shown in Figure 6.

In order to reveal the mechanism of the solute stationary phase interactions on the two columns the correlation of the slope and intercept values obtained on the two columns with various mobile phase mixtures (A, B and C) was also investigated. The correlation

TABLE 4.

The correlation coefficients of the slope (S) and the intercept ($\log k'_0$) values obtained on Sperisorb ODS and Supelcosil™ LC-ABZ column in the three mobile phase systems (A: MeOH - water; B: AcN - water, C: AcN, 0.1% TFA - water).

	Spherisorb ODS	Supelcosil™ LC ABZ
MeOH - Water	0.881	0.935
AcN - Water	0.999	0.939
AcN 0.1%TFA - Water	0.991	0.779

coefficients can be seen in Table 4. As it was observed earlier [6], the high correlation coefficient between the slope and intercept values shows higher similarity of the retention mechanism of the compounds. As it can be seen in Table 4, the correlation between the slope and the intercept values was high in almost every conditions. The compounds behaved as structurally unrelated in the Spherisorb ODS column with methanol and water as mobile phase, and on Suplecosil™ LC-ABZ column with acetonitrile, TFA and water mobile phase. It also can be seen that the extrapolated log k' to the zero organic phase concentrations are different obtained from the various mobile phase systems. The reason for that can be that mobile phase A and B did not contain buffer, so the actual pH could change by changing the organic modifier concentration, and the log k' vs organic phase plots could deviate from linearity around the 0% in a great extent. The mobile phase system C, which contained 0.1% TFA can be regarded as more defined one, as the compounds are in protonated form at every mobile phase composition.

TABLE 5.

The chromatographic hydrophobicity index values (φ_0) for the compounds investigated obtained in acetonitrile, 0.1% TFA and water mixtures.

Compound No.	Spherisorb ODS	Supelcosil™ LC-ABZ
1.	52.2	48.4
2.	51.5	47.7
3.	51.2	50.5
4.	51.4	42.8
5.	25.9	19.7
6.	30.8	20.6
7a	52.4	39.8
7b	56.8	42.5

For the characterization of the hydrophobicity of the compounds investigated we calculated the chromatographic hydrophobicity index as it was suggested recently [7]. The hydrophobicity index is ranging from 1 - 100 meaning the acetonitrile concentration by which the compounds have the retention time twice as much as the dead time. So the higher is the value the more hydrophobic the compound is. The calculated hydrophobicity index values obtained in the fully protonated system (acetonitrile, TFA, water) and in acetonitrile - water mixtures are listed in Table 5. The hydrophobicity index of the two columns show good correlation (the correlation coefficient is 0.92), although the compounds behaved slightly more hydrophilic on the Supelcosil™ LC-ABZ column than on the Spherisorb ODS.

CONCLUSIONS

In conclusion the effect of the free silanol groups could be eliminated on the specially treated reversed phase column. The log k' vs organic phase concentration plots were straight lines, while parabolic curves were obtained on the traditional reversed phase stationary phase. The separation of diastereomeric isomers could be carried out only on the Supelcosil™ LC-ABZ stationary phase at lower organic phase concentration. Poor separation of the isomers could be achieved on the traditional reversed-phase stationary phase as well utilizing the silanol effect at higher organic phase concentrations.

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